Cross-Reactive Protection against Enterohemorrhagic *Escherichia coli* Infection by Enteropathogenic *E. coli* in a Mouse Model[▽]

Carla Calderon Toledo,[1,2] Ida Arvidsson,[1] and Diana Karpman[1]*

Department of Pediatrics, Clinical Sciences Lund, Lund University, Lund, Sweden,[1] and Institute of Molecular Biology and Biotechnology, Universidad Mayor de San Andrés, La Paz, Bolivia[2]

Enteropathogenic *Escherichia coli* (EPEC) and enterohemorrhagic *E. coli* (EHEC) are related attaching and effacing (A/E) pathogens. The genes responsible for the A/E pathology are carried on a chromosomal pathogenicity island termed the locus of enterocyte effacement (LEE). Both pathogens share a high degree of homology in the LEE and additional O islands. EHEC prevalence is much lower in areas where EPEC is endemic. This may be due to the development of antibodies against common EPEC and EHEC antigens. This study investigated the hypothesis that EPEC infections may protect against EHEC infections. We used a mouse model to inoculate BALB/c mice intragastrically, first with EPEC and then with EHEC (*E. coli* O157:H7). Four control groups received either a nonpathogenic *E. coli* (NPEC) strain followed by EHEC (NPEC/EHEC), phosphate-buffered saline (PBS) followed by EHEC (PBS/EHEC), EPEC/PBS, or PBS/PBS. Mice were monitored for weight loss and symptoms. EPEC colonized the intestine after challenge, and mice developed serum antibodies to intimin and *E. coli* secreted protein B (encoded in the LEE). Prechallenge with an EPEC strain had a protective effect after EHEC infection, as only a few mice developed mild symptoms, from which they recovered. These mice had an increase in body weight similar to that in control animals, and tissue morphology exhibited mild intestinal changes and normal renal histology. All mice that were not prechallenged with the EPEC strain developed mild to severe symptoms after EHEC infection, with weight loss as well as intestinal and renal histopathological changes. These data suggest that EPEC may protect against EHEC infection in this mouse model.

Enterohemorrhagic *Escherichia coli* (EHEC) is a causative agent of diarrhea, hemorrhagic colitis, and hemolytic-uremic syndrome (HUS) (17). EHEC is characterized by the presence of Shiga toxin (Stx) as a major virulence factor (26). Enteropathogenic *Escherichia coli* (EPEC) is a leading cause of acute diarrhea among infants living under poor social conditions in developing countries (35). Typical EPEC is characterized by the presence of a virulence plasmid know as the EAF (EPEC adherence factor) (49). The EAF plasmid contains a cluster of genes encoding the bundle-forming pili (Bfp), required for localized adherence to epithelial cells (15). In contrast to EHEC, EPEC strains do not produce Stx.

Both pathogens induce characteristic attaching and effacing (A/E) lesions on intestinal enterocytes, characterized by intimate bacterial adhesion, destruction of microvilli, and accumulation of polymerized actin in pedestals beneath intimately attached bacteria (24). Bacterial factors required for the formation of the A/E lesion are encoded on a chromosomal pathogenicity island called the locus of enterocyte effacement (LEE) (12), which contains *eae*, encoding the adhesin intimin (22); *esc* and *sep*, encoding a type III secretion apparatus (12); and genes encoding proteins that are secreted via the type III secretion system, including *E. coli* secreted protein A (EspA), EspB, EspD (21), and the receptor for intimin (Tir) (29).

Intimate attachment of bacteria is mediated by intimin and its receptor translocated into host cells (29). EspA forms a filamentous organelle that acts as a channel through which bacterial proteins are transported into the eukaryotic cell (30). EspB and EspD form pores in the membranes of infected cells (19). EPEC and EHEC share a high degree of homology across the 41 genes contained in the LEE (39).

Epidemiological surveys regarding the prevalence of A/E pathogens revealed that EHEC infections are present mainly in developed countries and are not found frequently in developing countries, with the exception of Argentina (35). In Brazil, EPEC prevalence accounted for 33% of isolates among children younger than 2 years of age with diarrhea, and EHEC isolates were not detected (16, 43). The prevalence of A/E pathogens in Bolivia among children younger than 5 years of age with diarrhea was 7%, among which 95% of isolates corresponded to EPEC and 5% corresponded to EHEC (44).

The low prevalence of EHEC infections in developing countries may be explained by the development of antibodies against common EPEC and EHEC antigens by individuals living in areas where EPEC is endemic (34, 38). Several studies showed that children and adults develop an immune response against highly immunogenic virulence factors such as intimin and the Esps (7, 34, 38, 47), which are potential targets for vaccine development. In addition, IgA antibodies against intimin, Bfp, EspA, and EspB have been detected in colostrum samples from mothers living in areas where EPEC is endemic (33, 38), which may provide infants with effective protection against A/E pathogen infections (32).

This study used an established mouse model (5) to examine

* Corresponding author. Mailing address: Department of Pediatrics, Clinical Sciences Lund, Lund University, 22185 Lund, Sweden. Phone: 46-46-2220747. Fax: 46-46-2220748. E-mail: diana.karpman@med.lu.se.

TABLE 1. *E. coli* strains used in this study

Strain	Parental strain[a]	Category	Serotype	Presence of gene			Antibiotic resistance phenotype
				bfp	*eae*	*stx*$_2$	
73-1PB	73-1	EPEC	O127:H6	+	+	−	Amp^r Str^s
86-24PB	86-24	EHEC	O157:H7	−	+	+	Amp^s Str^r
Select96[b]		NPEC		−	−	−	Amp^r Str^s

[a] The parental strains were sensitive to ampicillin and streptomycin.
[b] Select96 competent cells from Promega.

the hypothesis that EPEC infection may have a protective effect against subsequent EHEC infection in mice.

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MATERIALS AND METHODS

Mice. BALB/c mice were bred in the animal facilities of the Department of Microbiology, Immunology and Glycobiology, Institute of Laboratory Medicine, Lund University. Male mice were used at 8 to 9 weeks of age.

Bacterial strains and cultures. Bacterial strains used in this study are listed in Table 1. EPEC strain 73-1 was isolated from the feces of a 2-year-old boy with diarrhea in La Paz, Bolivia, in 2004. The strain was typed for the O serogroup antigen and genotypically characterized for the presence of the *bfp*, *eae*, *stx*$_1$, and *stx*$_2$ genes (2, 18, 25). The strain corresponded to the O127:H6 serotype and was positive for the *bfp* and *eae* genes. The strain was found to be sensitive to ampicillin (Amp^s) and streptomycin (Str^s). Spontaneous ampicillin-resistant derivatives of this strain were developed as previously described (42). In order to enhance virulence, the resulting strain 73-1 (Amp^r Str^s) was first inoculated intragastrically into four ampicillin-treated BALB/c mice (1 g/liter ampicillin [Sigma-Aldrich, Stockholm, Sweden] in the drinking water 24 h before challenge and throughout the experiment). To confirm the colonization, fecal samples were collected 24, 48, 72, and 96 h after inoculation, plated on Luria broth (LB) agar supplemented with 50 μg/ml ampicillin, and analyzed by PCR for the detection of the *bfp* (18) and *eae* (25) genes. After 24 h, one mouse presented with a positive fecal culture for EPEC, and this strain was isolated, termed 73-1PB, and kept in LB-glycerol (85:15 [vol/vol]) at −80°C for further experiments. After 72 h, all fecal cultures from the four mice were found to be positive for EPEC by PCR. All mice presented some systemic symptoms, such as ruffled fur, from which they recovered by days 3 to 5.

EHEC strain 86-24 (*E. coli* O157:H7) was isolated during the Walla Walla, WA, outbreak of HUS and hemorrhagic colitis in November 1986 (48) and was kindly provided by A. D. O'Brien (Department of Microbiology and Immunology, Uniformed Services University of the Health Sciences, Bethesda, MD). This strain was previously characterized genotypically and phenotypically (28). The strain was found to be ampicillin and streptomycin sensitive. Spontaneous streptomycin-resistant derivatives of this strain were developed as previously described (42). To enhance virulence, the resulting strain 86-24 (Amp^s Str^r) was first inoculated into three streptomycin-treated BALB/c mice (5 g/liter streptomycin sulfate [MP Biomedicals, OH] in the drinking water 24 h before inoculation and throughout the experiment). *E. coli* O157:H7 was isolated on day 9 from colonic content of a sick mouse and detected by slide agglutination using an *E. coli* O157 latex test kit (Oxoid, Basingstoke, United Kingdom). The strain was termed 86-24PB and stored in LB-glycerol (85:15 [vol/vol]) at −80°C until use.

A laboratory *E. coli* strain, Select96 competent cells (Promega, Madison, WI), was used as a control strain. The strain has a plasmid mediating ampicillin resistance (pcDNA3; Invitrogen, Carlsbad, CA). The strain was genotypically characterized for the presence of *bfp*, *eae*, and *stx*$_2$ and found to be negative for these genes. The strain was termed nonpathogenic *E. coli* (NPEC).

For inoculation of mice, the EPEC, EHEC, and NPEC strains were grown overnight at 37°C in LB broth supplemented with 50 μg/ml ampicillin or 50 μg/ml streptomycin, as appropriate, and harvested by centrifugation. The pellet was washed in sterile phosphate-buffered saline (PBS) (pH 7.4; Medicago AB, Upp-

sala, Sweden) and resuspended in a solution of 20% (wt/vol) sucrose and 10% (wt/vol) NaHCO$_3$ in sterile water.

Infection protocol. Mice were divided into five groups, and each group received two inoculations (Table 2) at two different time points (Fig. 1).

For the first inoculation, mice were treated with ampicillin 24 h prior to inoculation, as described above, to reduce the commensal flora, thereby facilitating ampicillin-resistant strain (EPEC and NPEC) colonization of the gastro-intestinal tract. Mice were fasted for 16 h before inoculation. Mice were anesthetized with isoflurane (Forene; Abbott, Wiesbaden, Germany), and 100 μl of a bacterial suspension (10^9 CFU/ml) or sterile PBS was administered intragastrically through a soft polyethylene catheter (0.61-mm outer diameter; 0.28-mm inner diameter; Clay Adams, Parsippany, NJ) (28). After inoculation, the catheter was removed and food was provided *ad libitum*. During the course of infection, mice were monitored three to five times per day. The ampicillin treatment was discontinued after 7 days to enable recovery of the commensal flora.

In order to facilitate the clearing of bacteria from the first inoculation and to improve colonization of the EHEC strain, mice were treated with streptomycin from day 16 after the first inoculation until the end of the experiment (Fig. 1). Before the second inoculation, mice were fasted for 16 h. Under isoflurane anesthesia, 100 μl of a bacterial suspension (10^9 CFU/ml) or sterile PBS was administered intragastrically using a soft polyethylene catheter. Ten days after the second inoculation or when mice presented evident signs of disease, infected and control mice were sacrificed by cervical dislocation under isoflurane anesthesia, tissues were collected for histological examination, and a final disease score was given to each mouse as previously described (5) (Table 3). Symptom score 3 depicts the most severe clinical findings, as spontaneous death did not occur.

An attempt was made to perform the first inoculation without antibiotic treatment, but bacteria from the first challenge were not able to colonize efficiently. A second attempt was made to treat mice with streptomycin alone, using a streptomycin-resistant EPEC strain followed by the streptomycin-resistant EHEC strain. Under continuous streptomycin treatment, EPEC bacteria colonized the intestine persistently and were shed in the feces for up to 50 days after infection. Challenge with the streptomycin-resistant EHEC strain was performed at day 40, but under these conditions EHEC bacteria were unable to colonize. For these reasons, all experiments were carried out as described above, using ampicillin treatment for the ampicillin-resistant EPEC or NPEC strain followed by streptomycin treatment for the streptomycin-resistant EHEC strain. BALB/c mice were chosen for this study due to their high level of susceptibility to EHEC infection. Inoculation with EHEC under streptomycin treatment, without prior treatment with PBS or other bacterial strains, causes terminal illness in 100% of infected mice. All experiments were approved by the Animal Ethics Committee of Lund University.

Confirmation of colonization and bacterial shedding. To confirm the colonization of EPEC and NPEC strains, fecal samples were collected from days 1 to

TABLE 2. Inoculation groups in this study

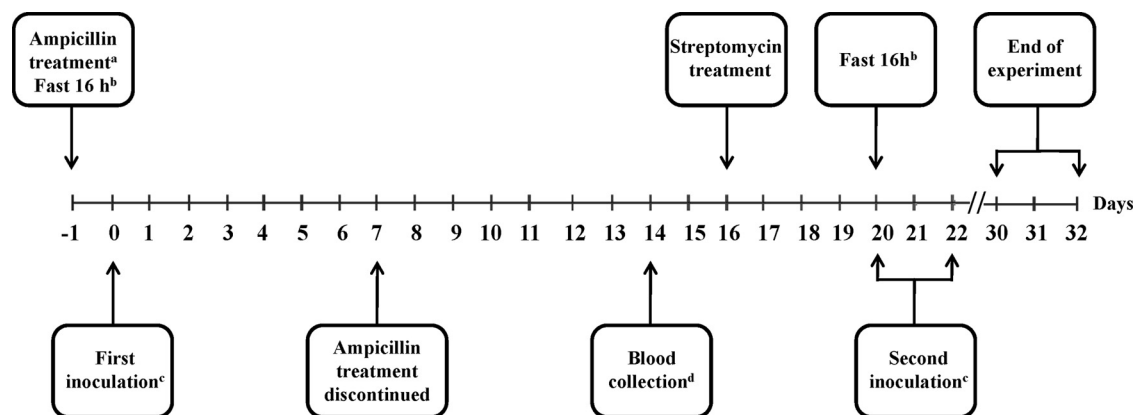| Group no.	No. of mice	First inoculum	Second inoculum	Group name
1	11	EPEC	EHEC	EPEC/EHEC
2	6	NPEC	EHEC	NPEC/EHEC
3	12	PBS	EHEC	PBS/EHEC
4	7	EPEC	PBS	EPEC/PBS
5	7	PBS	PBS	PBS/PBS

FIG. 1. Schematic presentation of infection protocol. Mice were initially inoculated with the EPEC or NPEC strain, followed 20 to 22 days later by a second inoculation with the EHEC strain. Antibiotics in drinking water, i.e., ampicillin and streptomycin, were used to enhance colonization of each strain but also to eradicate the first strain before the second inoculation. a, 24 h before inoculation; b, 16 h before inoculation; c, see Table 2 for the different inoculation groups; d, blood samples were also collected 6 days before the first inoculation.

3 after the first inoculation. To monitor bacterial shedding and to confirm clearance of bacteria after the first inoculation, fecal samples were also collected from days 7 to 20 (Fig. 1). Samples were plated on LB agar supplemented with 50 μg/ml ampicillin (for culture of both strains) and tested by PCR for the presence of the *bfp* and *eae* genes (for detection of EPEC).

Samples collected from the EPEC/EHEC, NPEC/EHEC, and EPEC/PBS groups (Table 2) on days 1 to 3 were positive for the EPEC or NPEC strain. In the EPEC/EHEC group, EPEC was cleared spontaneously (even before streptomycin treatment was initiated) in 5/11 mice 6 to 8 days before the second inoculation, and the remainder ($n = 6$) cleared the EPEC infection within 2 days after the start of streptomycin treatment. All mice in the NPEC/EHEC group cleared the NPEC infection before streptomycin treatment was given, 8 days before the second inoculation. In the EPEC/PBS group, all mice were positive for EPEC 8 days after inoculation with EPEC and cleared the EPEC infection within 3 days after the start of streptomycin treatment.

To confirm the colonization of EHEC, fecal samples were collected on days 1, 2, and 7 after the second inoculation, plated on LB agar supplemented with 50 μg/ml streptomycin, and tested for the presence of the O157 serogroup by slide agglutination. Samples from the EPEC/EHEC, NPEC/EHEC, and PBS/EHEC groups were positive for the EHEC strain at the three time points after the second inoculation.

Weight measurement. Body weight changes were calculated as percentages of initial body weight. For the five groups, weight was taken 1 day before the second inoculation, to account for initial body weight before fasting, and on a daily basis afterwards.

Antibody detection in serum samples. Blood samples were collected from the saphenous vein 6 days before and 14 days after the first inoculation of mice in the EPEC/EHEC group ($n = 11$), PBS/EHEC group ($n = 6$), and PBS/PBS group ($n = 4$), using capillary tubes for serum collection (Sarstedt, Nümbrecht, Germany). Serum samples were stored at −20°C until analysis.

The presence of IgM antibodies against EspB was detected by enzyme-linked immunosorbent assay (ELISA) as previously described (45). Briefly, plates were coated with 1/2,000 rabbit anti-EspB (45) overnight at 4°C, and the wells were

washed with PBS-Tween (Medicago, Uppsala, Sweden), blocked for 2 h with bovine serum albumin (Sigma-Aldrich, Stockholm, Sweden), washed, and incubated for 1 h with recombinant His-tagged EspB (45) (50 ng/well). After being washed, the wells were incubated with serum samples (1/50) for 2 h at 37°C. Wells were washed and incubated with peroxidase-conjugated goat anti-mouse IgM (1/500; Sigma-Aldrich) for 1.5 h. After being washed, wells were incubated for 30 min in the dark with an OPD solution (Dako, Glostrup, Denmark) and then resuspended in deionized water with the addition of H_2O_2 (30%), and the reaction was terminated with 0.5 M H_2SO_4. Absorbance (optical density at 490 nm [OD_{490}]) was measured at 490 nm. Changes in serum antibody levels were calculated as the percent increase/decrease compared to the initial absorbance values (before the first inoculation).

Isolation of EPEC and EHEC secreted proteins. EPEC (73-1PB) and EHEC (86-24PB) secreted proteins were isolated according to a previously published protocol (21), with the following modifications. Bacteria were grown in low-glucose Dulbecco's modified Eagle's medium (DMEM) (Gibco, Paisley, United Kingdom) to an OD_{600} of 1.0. Bacteria were pelleted by centrifugation at $10,000 \times g$ for 10 min, after which the supernatant was collected. Phenylmethylsulfonyl fluoride (50 μg/ml; Sigma-Aldrich) and EDTA (0.5 μM; Merck, Darmstadt, Germany) were added to the supernatant, which was passed through a 0.2-μm filter (Pall Corp., Ann Arbor, MI) and concentrated 330 times using IVD Ultracel 10K (Millipore, Carrigtwohill, Ireland).

Immunoblotting to detect antibodies to EPEC and EHEC secreted proteins. Secreted proteins from EPEC or EHEC (approximately 30 μg in each well) were run in a 10% Tris-HCl gel (Bio-Rad, Hercules, CA) and then transferred to a polyvinylidene difluoride (PVDF) membrane (Bio-Rad). Proteins were detected using goat anti-intimin (a gift from A. D. O'Brien) at 1:500, rabbit anti-EspB (27) at 1:5,000, or sera (1:500) from mice. The sera were collected before inoculation with EPEC as well as 14 days later. Bound antibodies were identified using polyclonal rabbit anti-goat immunoglobulin (Ig)–horseradish peroxidase (HRP) (1:500; Dako), polyclonal goat anti-rabbit Ig–HRP (1:1,000; Dako), or goat anti-mouse IgG–peroxidase (1:500; Sigma-Aldrich), respectively, and visualized using ECL Plus (GE Healthcare, Buckinghamshire, United Kingdom).

Histopathological analysis. Proximal and distal colon samples as well as kidneys were collected at the end of the experiment (10 days after the second inoculation or when evident signs of disease were observed after the second inoculation). Samples were fixed overnight in 4% paraformaldehyde (Sigma-Aldrich) and embedded in paraffin. Tissue sections (3 μm) were stained with hematoxylin-eosin (Merck) for kidneys and with periodic acid-Schiff stain for intestines. Stained tissue sections were then examined under an Axiostar Zeiss microscope mounted with an Axiocam MRc5 camera (Carl Zeiss, Göttingen, Germany). AxioVision AC software, version 4.4 (Carl Zeiss), was used for image processing. Samples were coded and examined in a blinded fashion. The degree of pathological findings was defined as mild, moderate, or severe.

Statistical analysis. Differences between the experimental groups regarding disease scores, body weight changes, and antibody levels were assessed by the Mann-Whitney U test. P values of ≤0.05 were considered significant. SPSS software, version 11 (SPSS, Chicago, IL), was used for statistical analyses.

TABLE 3. Symptom score descriptions

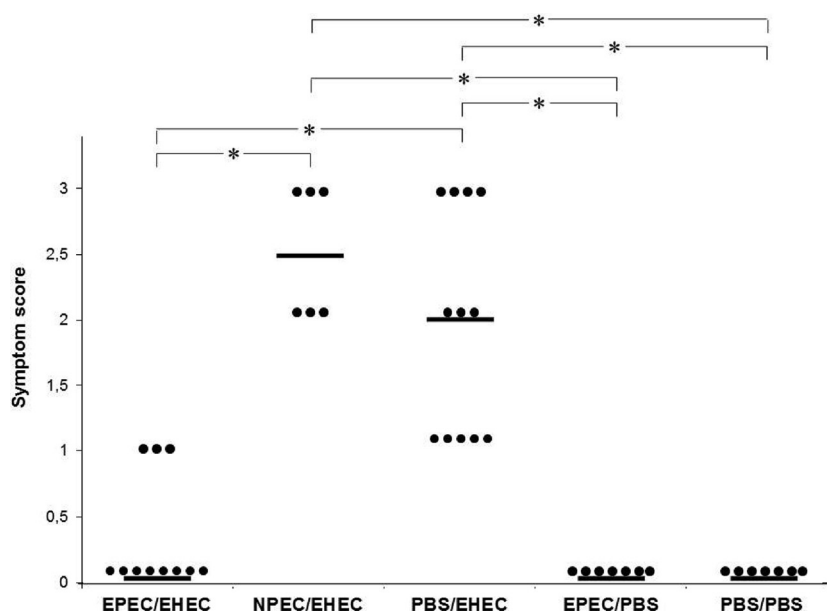| Score	Description	Clinical sign(s)
0	No clinical signs	
1	Mild clinical signs	Ruffled fur
2	Moderate clinical signs	Ruffled fur plus lethargy, hunched posture, decreased activity
3	Severe clinical signs	Paresis, paralysis, tremor, shivers, ataxia, terminal illness, severe weight loss (>20%)

FIG. 2. Symptom scores in mice. A final symptom score was assigned to each mouse after the second inoculation, as described in Table 3. The median value was calculated for each group (horizontal line). The highest symptom scores were found for mice from the NPEC/EHEC and PBS/EHEC groups, and there was no significant difference between these two groups. Comparison of symptoms in mice from the EPEC/EHEC group with those in the EPEC/PBS and PBS/PBS groups did not show a significant difference. ∗, significant difference between groups ($P < 0.01$).

RESULTS

Clinical signs of disease in the different inoculation groups. Mice were divided into five groups and received two separate inoculations (Table 2). After the first inoculation, mice from the EPEC/EHEC, NPEC/EHEC, and EPEC/PBS groups presented mild symptoms, such as ruffled fur, from which they recovered within 3 to 5 days. Symptoms were not observed in mice from the PBS/EHEC and PBS/PBS groups.

After the second inoculation, the different groups were compared with regard to the development of symptoms. All mice that did not receive a previous EPEC infection, i.e., mice in the NPEC/EHEC and PBS/EHEC groups, developed mild to severe symptoms after infection with the EHEC strain, and there was no significant difference ($P > 0.05$) between these two groups regarding symptom scores. Mice in the NPEC/EHEC and PBS/EHEC groups exhibited the highest symptom scores (Table 3). Terminally ill mice were found only in the PBS/EHEC group (4/12 [33.3%] mice). Although certain mice were severely ill, no spontaneous death occurred during the experiment. Mice in the EPEC/EHEC group exhibited lower symptom scores. Mild symptoms occurred in 3/11 (27%) mice, all of which recovered, and 8/11 (73%) mice in this group did not show any clinical signs of disease. There was no significant difference ($P > 0.05$) regarding symptom scores for mice in the EPEC/EHEC group compared with those in the EPEC/PBS or PBS/PBS group (Fig. 2).

Body weight changes during EHEC infection. Body weight changes were calculated as percentages of initial body weight for the five groups during the course of EHEC infection. After the second inoculation, mice in the NPEC/EHEC and PBS/EHEC groups exhibited weight loss, particularly during the first 2 days after inoculation, whereas mice in the EPEC/

EHEC, EPEC/PBS, and PBS/PBS groups recovered their initial body weight immediately after the initial fasting period. Body weight changes were expressed as a function of time (Fig. 3).

Antibody response to EspB in serum samples. Serum antibody levels were assessed by comparing individual values before the first inoculation with values 14 days after the first inoculation. At this time point, mice from the PBS/EHEC and PBS/PBS groups had received the same treatment (only PBS) and were therefore merged into one control group ($n = 10$) for comparison with mice in the EPEC/EHEC group ($n = 11$). Antibody levels against EspB were elevated in the EPEC/EHEC group, showing a median increase of 12.5% (range, 0 to 52.3%) 14 days after EPEC infection. As expected, there was no increase in anti-EspB levels in sera from mice that were treated with PBS. The increased anti-EspB level in the EPEC/EHEC group was statistically significant compared with the PBS/EHEC and PBS/PBS groups together ($P < 0.05$).

Cross-reactive antibody response to EHEC secreted proteins detected by immunoblotting. For the purpose of testing if mice exposed to EPEC developed an antibody response to EHEC secreted proteins, the latter were run in a gel and incubated with mouse sera taken before EPEC inoculation and 14 days later. Results showed that sera from 8/11 mice in the EPEC/EHEC group reacted with intimin and that 4/11 sera reacted with EHEC secreted protein B (EspB) after inoculation with EPEC and before inoculation with EHEC. A total of nine mice showed an antibody response to EHEC intimin and/or EspB. Bands were detected at approximately 94 kDa and 37 kDa, corresponding to intimin and EspB, respectively (Fig. 4, lanes 1 and 2) (27). No bands were visualized for sera taken from mice in the PBS/EHEC group ($n = 2$) before
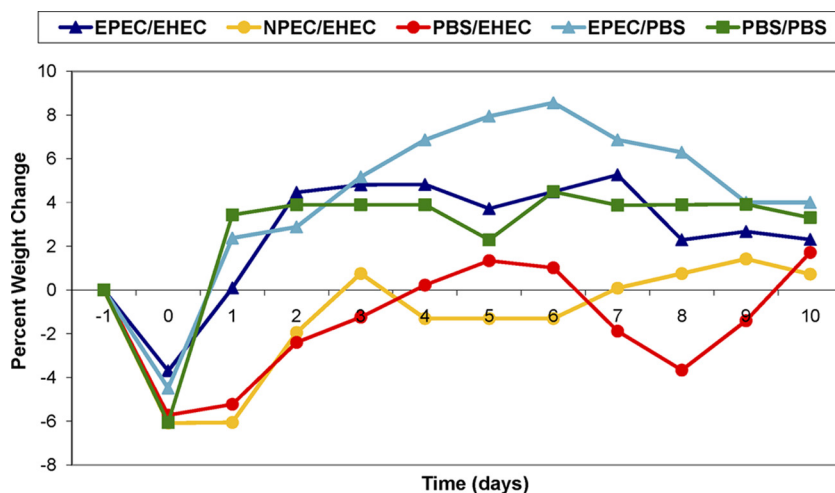
FIG. 3. Body weight changes during EHEC infection. Body weight changes were monitored for 11 days, starting 1 day before the second inoculation. Over the course of infection, mice from the NPEC/EHEC and PBS/EHEC groups exhibited weight loss and then regained their initial body weight toward the end of the observation period. Mice from the EPEC/EHEC, EPEC/PBS, and PBS/PBS groups recovered their initial body weight after the fasting period and exhibited increases in body weight. Symbols at each time point represent the average values for the groups. Significant differences ($P < 0.01$) in body weight changes were found in comparing the following groups: EPEC/EHEC with NPEC/EHEC, EPEC/EHEC with PBS/EHEC, NPEC/EHEC with PBS/PBS, NPEC/EHEC with EPEC/PBS, PBS/EHEC with PBS/PBS, PBS/EHEC with EPEC/PBS, and PBS/PBS with EPEC/PBS ($P < 0.05$ for the last comparison). No significant differences ($P > 0.05$) in body weight changes were found in comparing the following groups: EPEC/EHEC with EPEC/PBS, EPEC/EHEC with PBS/PBS, and NPEC/EHEC with PBS/EHEC.

inoculation with EHEC or for those from mice in the PBS/PBS group ($n = 3$). Similar results were obtained using an extract of secreted proteins from the EPEC strain (Fig. 4, lanes 3 and 4). As a positive control, the EHEC secreted proteins reacted with rabbit anti-EspB (Fig. 4, lane 5) and goat anti-intimin (Fig. 4, lane 6).

Intestinal and renal pathology in the different inoculation groups. Intestines and kidneys from mice were coded for blind assessment and examined by light microscopy for histopathological lesions, which are summarized according to severity in Table 4. Changes consisted of inflammatory infiltrates, lymph node hyperplasia, thickening of the submucosa, edema, and goblet cell depletion (Fig. 5). The most severe intestinal changes were found in mice from the NPEC/EHEC and PBS/EHEC groups. Few pathological changes were found in mice from the EPEC/EHEC and EPEC/PBS groups. No alterations in the intestinal structure were noted for the PBS/PBS group.

Renal pathology was demonstrated mainly for mice in the PBS/EHEC group. Pathological changes in this group included tubular cell desquamation, dilated tubular structures, glomerular capillary congestion and occlusion, and red blood cells in tubular lumina. Tubular desquamation and dilated tubuli were also demonstrated for mice from the NPEC/EHEC group. Mice from the EPEC/EHEC, EPEC/PBS, and PBS/PBS groups did not exhibit renal pathology (Fig. 6 shows images for EPEC/EHEC and PBS/PBS mice).

DISCUSSION

Due to the severity of disease, clinical trials using wild-type *E. coli* O157:H7 strains cannot be performed on human volunteers. A mouse model has been developed (5, 51) which mimics certain aspects of severe human *E. coli* O157:H7 infection, such as severe systemic and neurological symptoms as

well as pronounced pathology of the gastrointestinal tract and kidneys. Mice develop marked tubular damage as well as decreased renal function and thrombocytopenia resembling certain aspects of human HUS (5). Since it has been hypothesized that EPEC infection may confer immunity against EHEC infection in areas of endemicity (37, 38), we tested this in the mouse model. Prechallenge with an EPEC strain protected mice from the symptoms and pathology associated with EHEC infection. The degree of homology between these two A/E pathogens suggests that a protective immune response may occur. Indeed, results showed that mice developed antibodies to intimin and EspB after the EPEC infection, indicating an immune response to EPEC virulence factors. An antibody response was also mounted to EHEC intimin and EspB, even before inoculation with EHEC, suggesting cross-reactivity. Due to the similarity between the strains, there may be multiple protective mechanisms conferring cross-reactive immunity between EPEC and EHEC pathogens.

EPEC and EHEC share a high degree of homology across the genes carried on the LEE pathogenicity island (39). The LEE represents only one such island. In addition, comparison of 177 O islands showed that 69 islands shared >90% nucleotide homology between EHEC O157:H7 (EDL933) and EPEC O127:H6 (2348/69) (46). Moreover, phylogenetic analysis suggested that EHEC O157:H7 may have evolved from EPEC strains that acquired phage-encoded Stx (13, 52) or that the strains developed in parallel, acquiring similar virulence factors (41).

A protective effect among A/E pathogens was suggested in a study in which challenge of rabbits with a Shiga toxin-producing RDEC-1 strain possessing truncated intimin but retaining immunogenicity had a protective effect when the same rabbits were subsequently challenged with the wild-type strain (1). Antibodies to intimin have been detected during and after
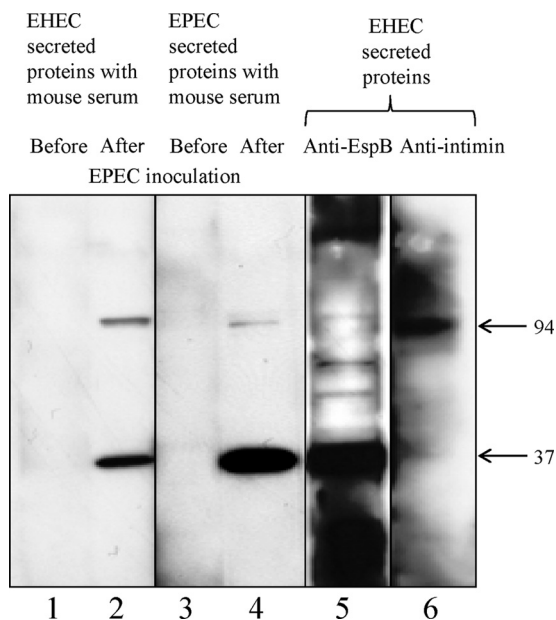
FIG. 4. Cross-reactive antibody response to EHEC and EPEC secreted proteins in mouse sera. EHEC secreted proteins were blotted with mouse sera collected before (lane 1) and after (lane 2) EPEC inoculation, and the blot shows the development of antibodies reacting with EHEC intimin and EspB. EPEC secreted proteins were blotted with mouse sera collected before (lane 3) and after (lane 4) EPEC inoculation, and the blot shows the development of antibodies to EPEC intimin and EspB. EHEC secreted proteins reacted with anti-EspB at 37 kDa (lane 5) and with anti-intimin at 94 kDa (lane 6). All lanes were run in the same gel.

EPEC or EHEC infections (11, 27, 31). It has been suggested that these antibodies could confer protection against subsequent EPEC infection. This could not be confirmed, however, in a homologous rechallenge setting in which human volunteers were first challenged with EPEC strains (wild-type *E. coli* O127:H6 or its corresponding isogenic Δ*eae* mutant) and then rechallenged with the wild-type strain (11). No correlation was found between anti-intimin antibodies and severity of disease, although volunteers prechallenged with the wild-type strain developed fewer symptoms than those prechallenged with the Δ*eae* mutant. In the same study, heterologous rechallenge with EPEC strains also failed to induce preventive immunity, determined by the clinical end point of diarrhea in human volunteers. The reduction in severity of symptoms after homologous challenge was thus attributed to an antibody response to O antigens. These studies indicated that protection was serotype specific. The protective effect demonstrated here would most probably not have been mediated by an acquired immune response to lipopolysaccharide (LPS), since the EPEC and EHEC strains studied do not share the same LPS serogroup.

Mice in the NPEC/EHEC group exhibited milder renal pathology than mice in the PBS/EHEC group after EHEC infection. This may indicate that a previous challenge with a nonpathogenic *E. coli* strain may have some degree of protective effect, although not to the same extent as the protective effect mediated by a previous challenge with an EPEC strain. We speculate that a possible protective effect might be explained partly by the "endotoxin tolerance" mechanism, by which a previous challenge with LPS could invoke insensitivity upon a second challenge as a response from the organism to regulate excessive inflammation that may be deleterious (3). This phenomenon could last for hours to days, after which typical proinflammatory conditions would recur upon endotoxin stimulation (3, 4), suggesting that such an effect would have to last for several days after the first challenge to play a role in the present mouse model. We have shown that bacteria from the first challenge were eradicated at least 4 to 8 days before the second bacterial infection. In particular, the NPEC strain was eradicated 8 days before the second inoculation. We thus cannot exclude the possibility that endotoxin tolerance or the presence of other shared *E. coli* surface antigens could

TABLE 4. Histopathological findings for the different inoculation groups[a]

Pathological finding	EPEC/EHEC (n = 5 intestines and 10 kidneys)	NPEC/EHEC (n = 6 intestines and 6 kidneys)		PBS/EHEC (n = 6 intestines and 10 kidneys)			EPEC/PBS (n = 7 intestines and 7 kidneys)
	Mild	Mild	Moderate	Mild	Moderate	Severe	Mild
Intestines							
Inflammatory infiltrates	3	2	2	4	1		1
Lymph node hyperplasia			5		1		2
Crypt hyperplasia		1	1				
Goblet cell depletion	1	2		3		1	
Thickening of the submucosa	1		4	3			
Shrunken interstitial space			1	1			
Edema		3					
Kidneys							
Tubular desquamation		4		4	1	1	
Glomerular congestion				1	1		
RBCs in the tubular lumen				1			

[a] Tissues were obtained at the end of the experiment (10 days after the second inoculation or when evident signs of disease were observed after the second inoculation). Tissues from mice in the PBS/PBS group (n = 7 intestines and 7 kidneys) did not exhibit any histopathological changes. Tissues from mice in the EPEC/EHEC and EPEC/PBS groups showed only mild changes. Tissues from mice in the NPEC/EHEC group showed only mild and moderate changes. RBCs, red blood cells.
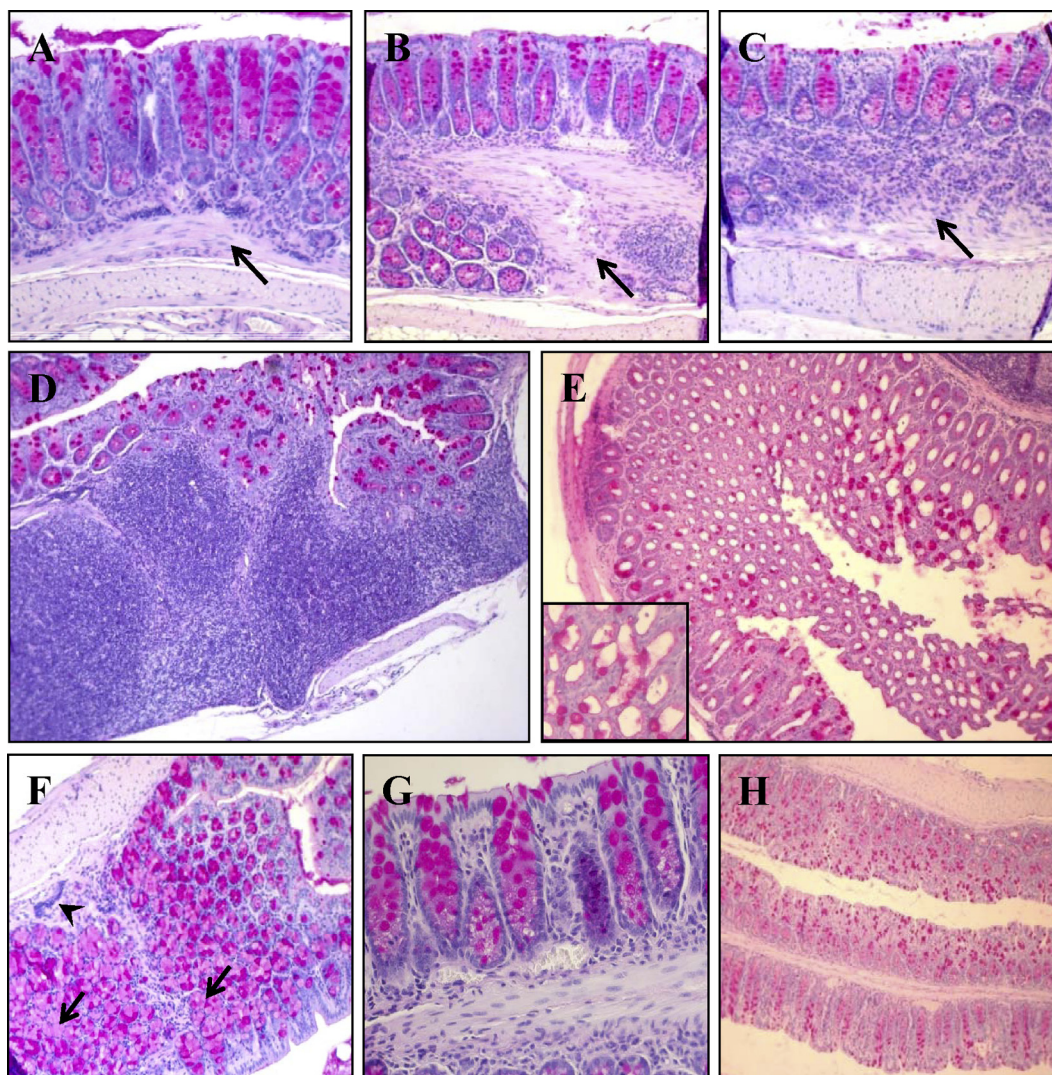
FIG. 5. Intestinal pathology in mice after the second inoculation. Distal colons of mice from the EPEC/EHEC (A), NPEC/EHEC (B), and PBS/EHEC (C) groups show inflammatory infiltrates and thickening of the submucosa (arrows). (D) Distal colon from a mouse in the NPEC/EHEC group showing lymph node hyperplasia. (E) Distal colon from a mouse in the PBS/EHEC group showing goblet cell depletion. See the inset for a magnified view. (F) Proximal colon from a mouse in the PBS/EHEC group showing shrunken interstitial space (arrows) and interstitial infiltrates (arrowhead). (G) Distal colon from a mouse in the EPEC/PBS group showing normal histology. Magnification, ×400. (H) Distal colon from a mouse in the PBS/PBS group showing normal histology. Magnification for all panels except for panel G, ×100.

have had a minor impact regarding protection from the severity of EHEC-induced pathology.

Before EHEC infection, mice were treated with streptomycin to remove EPEC bacteria from the gut. Interestingly, while developing the infection protocol, we observed that when EPEC bacteria colonized the gut persistently, EHEC bacteria were incapable of colonizing the gut and mice did not develop any symptoms. This phenomenon could also play a protective role against EHEC infection in individuals living in areas where EPEC is endemic. The mild histopathological changes found in the intestines of mice in the EPEC/PBS group may explain why mice in the EPEC/EHEC group also had mild intestinal histopathological changes: this may be residual EPEC-mediated damage not related to EHEC infection.

Most human EPEC strains, including the one used in this study, express intimin type α, while intimin γ is associated

mainly with EHEC serotypes, including O157:H7 (36). Intestinal tissue tropism may be determined in part by the intimin type (14, 50). Studies using a prototypical EPEC strain showed adhesion to the proximal and distal human small intestine and to follicle-associated epithelium (FAE) of Peyer's patches but showed limited adhesion to human colonic samples (40). It is believed that EHEC binds FAE and villi of the terminal ileal region (8, 40) and subsequently colonizes the human colon. As described above, in developing the present infection protocol, when EPEC bacteria colonized the guts of mice persistently, EHEC bacteria were unable to colonize. This may indicate that EPEC and EHEC compete for common loci of colonization. The infection protocol used in this study included the use of antibiotics to ensure that EPEC bacteria from the first challenge were eradicated before the second challenge with EHEC, and therefore the tissue tropisms of both strains de-
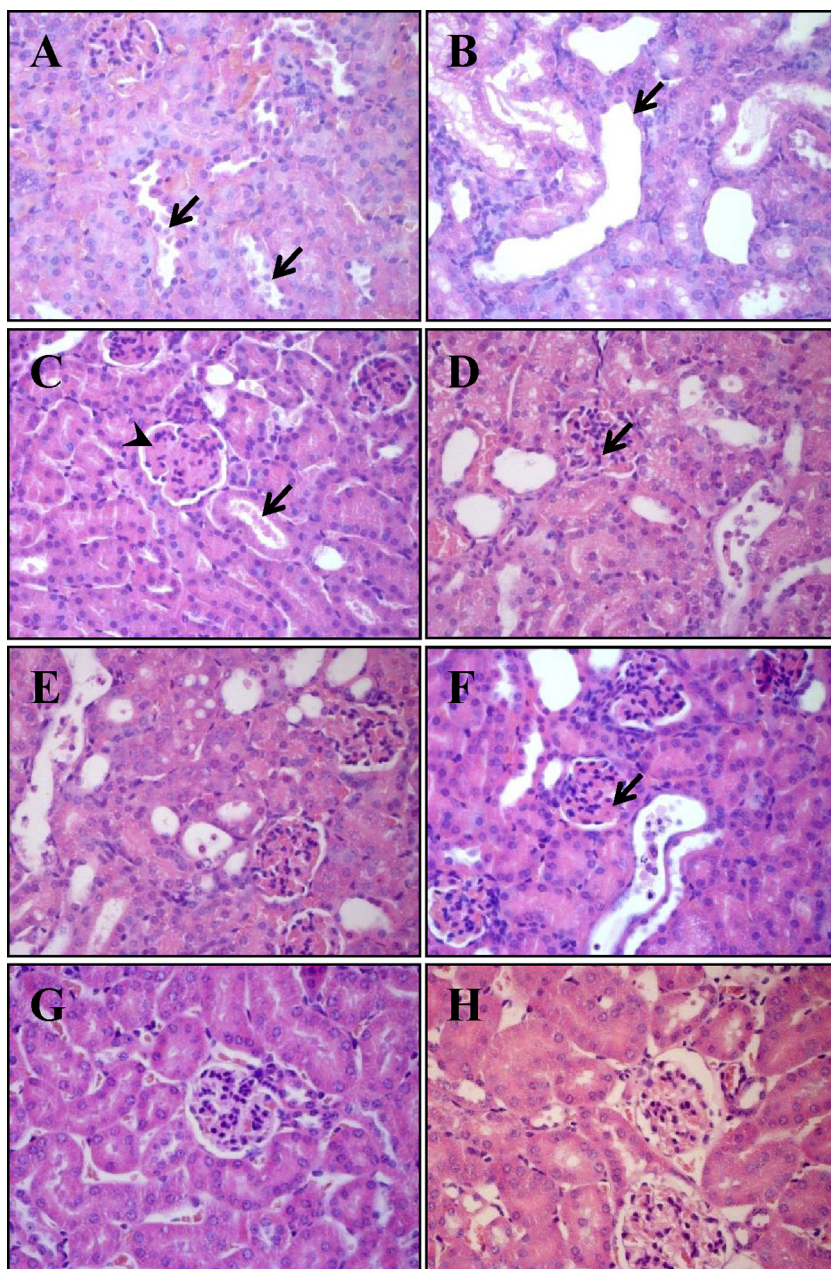
FIG. 6. Renal pathology in mice after the second inoculation. Panels A and B show tissues taken from mice in the NPEC/EHEC group. (A) Renal cortex showing tubular desquamation (arrows). (B) Dilated tubuli in the renal cortex (arrow). Panels C, D, E, and F show tissues obtained from mice in the PBS/EHEC group. (C) Renal cortex showing red blood cells in tubuli (arrow). (D, E, and F) Massive tubular desquamation as tubular structures are denuded of cells. Glomerular capillary congestion and occlusion are demonstrated (the arrow in panel D shows congestion and that in panel F shows occlusion; the arrowhead in panel C shows occlusion). Renal specimens taken from mice in the EPEC/EHEC (G) and PBS/PBS (H) groups showed normal histology. Magnification, ×400.

termining colonization sites most probably did not play a role regarding the protective effect observed. We believe that the protective effect was mediated by a humoral immune response to common EPEC and EHEC antigens.

The development of antibodies against Esp proteins in serum, saliva, colostrum, and breast milk during EPEC infection has been reported before (7, 33, 34, 37, 38, 47) and is consistent with our findings. Younger children have a higher propensity for symptomatic EPEC infection (35, 49), most probably due

to the fact that they have not developed a sufficient immune response. Breast-feeding has a protective effect against EPEC infection, and breast milk contains antibodies capable of preventing EPEC adherence (6, 9). In the present study, we showed that mice developed immunity against intimin and EspB, and we speculate that the protection observed may be mediated by a complex immune response against a wider variety of factors. Nonetheless, EspB was shown to be important in mediating diarrhea in human volunteers who developed an antibody response

to a wild-type EPEC strain. Volunteers who ingested an Δ*espB* mutant strain developed fewer symptoms (47).

Animal studies with mice and piglets have suggested the use of intimin vaccines (10, 23). The results of this study may be relevant for the development of live vaccines against EHEC infection based on the closely related A/E pathogen EPEC with attenuated virulence. Furthermore, the use of live vaccines would promote an efficient immune response against a broad range of known virulence determinants as well as a number of as yet unidentified virulence factors shared by both EHEC and EPEC, in contrast to purified vaccines based on only one or two virulence factors.

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